

File Number : 97-15C5
Filing Date: February 5, 2004
Express Mail Label No. EL988277935US

UNITED STATES PATENT APPLICATION

OF

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FOR

MAMMALIAN CYTOKINE-LIKE FACTOR 7

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This application is a continuation of U.S. Application Serial No. 09/633,452, filed August 7, 2000, which is a continuation of U.S. Application Serial No. 09/066,745, filed April 24, 1998, which claims the benefit of U.S. Provisional Application Serial No. 60/071,676 filed January 16, 1998; and U.S. Provisional Application Serial No. 60/044,886 filed April 25, 1997.

BACKGROUND OF THE INVENTION

Proliferation and differentiation of cells of multicellular organisms are controlled by hormones and polypeptide growth factors. These diffusible molecules allow cells to communicate with each other and act in concert to form cells and organs, and to repair and regenerate damaged tissue. Examples of hormones and growth factors include the steroid hormones (e.g. estrogen, testosterone), parathyroid hormone, follicle stimulating hormone, the interleukins, platelet derived growth factor (PDGF), epidermal growth factor (EGF), granulocyte-macrophage colony stimulating factor (GM-CSF), erythropoietin (EPO) and calcitonin.

Hormones and growth factors influence cellular metabolism by binding to proteins. Proteins may be integral membrane proteins that are linked to signaling pathways within the cell, such as second messenger systems. Other classes of proteins are soluble molecules, such as the transcription factors.

Of particular interest are cytokines, molecules that promote the proliferation and/or differentiation of cells. Examples of cytokines include erythropoietin (EPO), which stimulates the development of red blood cells; thrombopoietin (TPO), which stimulates development of cells of the megakaryocyte lineage; and granulocyte-colony stimulating factor (G-CSF), which stimulates development of neutrophils. These cytokines are useful in restoring normal blood cell levels in patients suffering from anemia or receiving chemotherapy for cancer. The demonstrated *in vivo* activities of these cytokines illustrates the enormous clinical potential of, and need for, other cytokines, cytokine agonists, and cytokine antagonists.

SUMMARY OF THE INVENTION

The present invention addresses this need by providing a novel polypeptide called cytokine-like factor 7, hereinafter referred to as Zcyto7 and related 5 compositions and methods.

Thus, one aspect of the present invention provides for an isolated Zcyto7 polypeptide having amino acid sequences as follows. Both the human and the mouse cDNAs have been discovered. The human sequences are defined by SEQ ID NOs: 1 10 and 2. The murine nucleotide and amino acid sequences are defined by SEQ ID NOs: 11 and 12.

The nucleotide sequence of SEQ ID NO:1 contains an open reading frame encoding a polypeptide of about 180 amino acids with the initial Met as shown in 15 SEQ ID NO:1 and SEQ ID NO:2. A predicted signal sequence is comprised of amino acid residues 1-20, and the resultant predicted mature Zcyto7 polypeptide is represented by the amino acid sequence extending from amino acid residue 21, a glutamine to and including amino acid residue 180 a phenylalanine, also represented by SEQ ID NO: 14. Peptide mapping data indicate that mature Zcyto7 can be comprised of a number of N- 20 terminal mature variants including the amino acid sequence extending from amino acid residue 23, an arginine to and including amino acid residue 180 of SEQ ID NO: 2, also defined by SEQ ID NO: 36; amino acid sequence extending from amino acid residue 27, a serine to and including amino acid residue 180 of SEQ ID NO: 2, also defined by SEQ ID NO: 37; the amino acid sequence defined by amino acid residue 30, a lysine, to 25 and including amino acid residue 180 of SEQ ID NO: 2, also defined by SEQ ID NO: 38; amino acid sequence extending from amino acid 28, a lysine, to and including amino acid residue 180 of SEQ ID NO:2, also defined by SEQ ID NO: 41 and the amino acid sequence extending from amino acid residue 53, a methionine, to and including amino acid residue 180, also defined by SEQ ID NO: 42. The only observed 30 cleavage at the carboxyl terminus is the phenylalanine at position 180 can be cleaved off. This can occur in all of the above-defined mature Zcyto7 polypeptides an example of which is shown by SEQ ID NO:43. Additional variants of human Zcyto7 are defined by SEQ ID NOs: 15-25. Within an additional embodiment, the polypeptide further comprises an affinity tag.

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SEQ ID NOs: 11 and 12 define murine Zcyto7 wherein the mature protein extends from amino acid residues amino acid residue 21, a histidine, to and

including amino acid residue 180 a phenylalanine, also defined by SEQ ID NO: 39; or as an alternative splice site from amino acid residue 23, an arginine, to and including amino acid 180 also defined by SEQ ID NO: 40. The present invention is also comprised of polypeptides having an amino acid sequence at least 90% identical, more 5 preferably 95%, 97% or 99% identical to those Zcyto7 polypeptides defined in above.

An additional embodiment of the present invention relates to a peptide or polypeptide which has the amino acid sequence of an epitope-bearing portion of a Zcyto7 polypeptide having an amino acid sequence described above. Peptides or 10 polypeptides having the amino acid sequence of an epitope-bearing portion of a Zcyto7 polypeptide of the present invention include portions of such polypeptides with at least nine, preferably at least 15 and more preferably at least 30 to 50 amino acids, although epitope-bearing polypeptides of any length up to and including the entire amino acid sequence of a polypeptide of the present invention described above are also included in 15 the present invention. Examples of said polypeptides are defined by the amino acid sequences of SEQ ID NOs: 25-35. Also claimed are any of these polypeptides that are fused to another polypeptide or carrier molecule.

The present invention further comprises a polypeptide defined by SEQ 20 ID NOs: 15-25 wherein the amino termini of said polypeptides are modified and begin at either amino acid residue 3, an arginine; amino acid residue 7, a serine; amino acid residue 8, a lysine; amino acid residue 10, a lysine or amino acid residue 33 methionine.

The present invention is further comprised of a polypeptide wherein the 25 polypeptide is a polypeptide defined by SEQ ID NOs: 2, 12, 14-25 and 36 to 42 wherein the amino acid sequences end at the isoleucines at amino acid residue 179 of SEQ ID NO: 2, at amino acid residue 159 of SEQ ID NOs: 14-25, which corresponds to amino acid residue 157 of SEQ ID NO:36, amino acid residue 153 of SEQ ID NO:37, amino acid residue 150 of SEQ ID NO:38, amino acid residue 159 of SEQ ID NO: 39, 30 amino acid residue 157 of SEQ ID NO:40, amino acid residue 152 SEQ ID NO:42, amino acid residue 127 of SEQ ID NO:42.

The present invention is further comprised of an isolated peptide or 35 polypeptide of the above-described peptides or polypeptide having an amino acid sequence modified by addition, deletion and/or replacement of one or more amino acid residues and which maintains the biological activity of said peptide or polypeptide.

Within a further aspect of the invention there is provided a chimeric polypeptide consisting essentially of a first portion and a second portion joined by a peptide bond. The first portion of the chimeric polypeptide consists essentially of (a) a 5 Zcyto7 polypeptide as described above (b) allelic variants of the polypeptides described above. The second portion of the chimeric polypeptide consists essentially of another polypeptide such as an affinity tag. Within one embodiment the affinity tag is an immunoglobulin F_C polypeptide. The invention also provides expression vectors 10 encoding the chimeric polypeptides and host cells transfected to produce the chimeric polypeptides.

Another aspect of the present invention provides for isolated nucleic acid molecules comprising a polynucleotide selected from the group consisting of: (a) a 15 nucleotide sequence encoding the Zcyto7 polypeptides described above; (b) a nucleotide sequence encoding the polypeptides of SEQ ID NOs: 14-40 and (c) a nucleotide sequence complementary to any of to any of the nucleotide sequences in (a) or (b).

Further embodiments of the invention include isolated nucleic acid molecules that comprise a polynucleotide having a nucleotide sequence at least 90% 20 identical, and more preferably 95%, 97%, 98%, or 99% identical to any of the nucleotide sequences in (a), (b) or (c) above, or a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence of (a) (b) or (c) above. An additional nucleic acid embodiment of the present invention relates to an isolated nucleic acid molecule comprising an amino acid of an 25 epitope-bearing portion of a Zcyto7 polypeptide.

Within another aspect of the invention there is provided an expression vector comprising (a) a transcription promoter; (b) a DNA segment encoding a 30 polypeptide described above, and (c) a transcription terminator, wherein the promoter, DNA segment, and terminator are operably linked.

Within a third aspect of the invention there is provided a cultured eukaryotic cell into which has been introduced an expression vector as disclosed above, wherein said cell expresses a protein polypeptide encoded by the DNA segment.

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In another embodiment of the present invention is an isolated antibody that binds specifically to a Zcyto7 polypeptide described above. Also claimed is a

method for producing antibodies which bind to a Zcyto7 polypeptide comprising inoculating a mammal with a Zcyto7 polypeptide or Zcyto7 epitope-bearing polypeptide so that the mammal produces antibodies to the polypeptide; and isolating said antibodies.

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These and other aspects of the invention will become evident upon reference to the following detailed description.

DETAILED DESCRIPTION OF THE INVENTION

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The teachings of all of the references cited herein are incorporated in their entirety herein by reference.

The term "affinity tag" is used herein to denote a polypeptide segment 15 that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A [Nilsson *et al.*, *EMBO J.* 4:1075 (1985); Nilsson *et al.*, 20 *Methods Enzymol.* 198:3 (1991)], glutathione S transferase [Smith and Johnson, *Gene* 67:31 (1988)], Glu-Glu affinity tag [Grussenmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7952-4 (1985)], substance P, FLAG™ peptide (Hopp *et al.*, *Biotechnology* 6:1204-10 (1988), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford *et al.*, *Protein Expression and Purification* 2: 95-107 (1991). 25 DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

The term "allelic variant" denotes any of two or more alternative forms 30 of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

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The term "expression vector" denotes a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments may

include promoter and terminator sequences, and may optionally include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

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The term "isolated", when applied to a polynucleotide molecule, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are 10 those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art. See for example, 15 Dynan and Tijan, *Nature* 316:774-78 (1985). When applied to a protein, the term "isolated" indicates that the protein is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated protein is substantially free of other proteins, particularly other proteins of animal origin. It is preferred to provide the protein in a highly purified form, i.e., 20 greater than 95% pure, more preferably greater than 99% pure.

The term "operably linked", when referring to DNA segments, denotes that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in the promoter and proceeds through the coding 25 segment to the terminator

The term "polynucleotide" denotes a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, 30 synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules.

The term "complements of polynucleotide molecules" denotes polynucleotide molecules having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' 35 ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and 5 GAC triplets each encode Asp).

The term "promoter" denotes a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-10 coding regions of genes.

The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in 15 which it is synthesized. The larger peptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule (i.e., a ligand) and mediates the effect of the ligand on the cell. 20 Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration 25 in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. Most nuclear receptors also exhibit a multi-domain structure, including an amino-terminal, 30 transactivating domain, a DNA binding domain and a ligand binding domain. In general, receptors can be membrane bound, cytosolic or nuclear; monomeric (e.g., thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (e.g., PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

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The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions.

For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of

5 the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of $<10^9 \text{ M}^{-1}$.

A "soluble protein" is a protein polypeptide that is not bound to a cell membrane.

10 Within preferred embodiments of the invention the isolated polynucleotides will hybridize to similar sized regions of the DNA of SEQ ID NO:1, or a sequence complementary thereto, under stringent conditions. In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for

15 the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typical stringent conditions are those in which the salt concentration is about 0.02 M or less at pH 7 and the temperature is at least about 60°C.

20 As previously noted, the isolated polynucleotides of the present invention include DNA and RNA. Methods for isolating DNA and RNA are well known in the art. Total RNA can be prepared using guanidine HCl extraction followed by isolation by centrifugation in a CsCl gradient [Chirgwin *et al.*, *Biochemistry* 18:52-94 (1979)]. Poly (A)⁺ RNA is prepared from total RNA using the method of Aviv and Leder, *Proc. Natl. Acad. Sci. USA* 69:1408-1412 (1972). Complementary DNA (cDNA) is prepared from poly(A)⁺

25 RNA using known methods. Polynucleotides encoding Zcyto7 polypeptides are then identified and isolated by, for example, hybridization or PCR.

30 Additionally, the polynucleotides of the present invention can be synthesized using a DNA synthesizer. Currently the method of choice is the phosphoramidite method. If chemically synthesized double stranded DNA is required for an application such as the synthesis of a gene or a gene fragment, then each complementary strand is made separately. The production of short genes (60 to 80 bp) is technically straightforward and can be accomplished by synthesizing the complementary strands and then annealing them. For the production of longer genes

35 (>300 bp), however, special strategies must be invoked, because the coupling efficiency of each cycle during chemical DNA synthesis is seldom 100%. To overcome this problem, synthetic genes (double-stranded) are assembled in modular form from single-

stranded fragments that are from 20 to 100 nucleotides in length. In addition to the protein coding sequence, synthetic genes can be designed with terminal sequences that facilitate insertion into a restriction endonuclease sites of a cloning vector and other sequences should also be added that contain signals for the proper initiation and 5 termination of transcription and translation.

See Glick, Bernard R. and Jack J. Pasternak, *Molecular Biotechnology, Principles & Applications of Recombinant DNA*, (ASM Press, Washington, D.C. 1994), Itakura, K. *et al.* Synthesis and use of synthetic oligonucleotides. *Annu. Rev. Biochem.* 10 53 : 323-356 (1984), and Climie, S. *et al.* Chemical synthesis of the thymidylate synthase gene. *Proc. Natl. Acad. Sci. USA* 87 :633-637 (1990).

Those skilled in the art will recognize that the sequences disclosed in SEQ ID NOS:1 and 2 represent a single allele of the human. There are a number of 15 naturally occurring mature N-terminal variants having the leader sequence cleaved at differing positions. They include the sequences defined by SEQ ID NOS 14, 36, 37 and 38. Allelic variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Examples of variants of human Zcyto7 are represented by the polypeptides of SEQ ID NOS: 15-25.

20 The murine Zcyto7 cDNA and protein are disclosed by SEQ ID NOS: 11 and 12. The mature Zcyto7 polypeptide is defined by SEQ ID NOS: 39 and 40.

The present invention further provides counterpart proteins and 25 polynucleotides from other species ("species orthologs"). Of particular interest are Zcyto7 polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primates. Species orthologs of the human Zcyto7 protein can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a 30 cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses the protein. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A protein-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human or mouse 35 cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent No. 4,683,202), using primers designed from the sequences disclosed herein.

Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to the protein. Similar techniques can also be applied to the isolation of genomic clones. As used and claimed the language "an isolated polynucleotide which encodes a 5 polypeptide, said polynucleotide being defined by SEQ ID NO: 2" includes all allelic variants and species orthologs of the polypeptide of SEQ ID NO:2.

The present invention also provides isolated protein polypeptides that are substantially homologous to the protein polypeptides of SEQ ID NO: 2 and its 10 species orthologs. By "isolated" is meant a protein or polypeptide that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably 15 greater than 99% pure. The term "substantially homologous" is used herein to denote polypeptides having 50%, preferably 60%, more preferably at least 80%, sequence identity to the sequence shown in SEQ ID NO:2, or its species orthologs. Such polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO:2, or its species orthologs. Percent sequence identity is 20 determined by conventional methods. See, for example, Altschul *et al.*, *Bull. Math. Bio.* 48: 603-616 (1986) and Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915-10919 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "blossom 62" scoring matrix of Henikoff and Henikoff (*ibid.*) as shown in Table 2 25 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{[\text{length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences}]} \times 100$$

Table 2

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
5	A	4																		
	R	-1	5																	
	N	-2	0	6																
10	D	-2	-2	1	6															
	C	0	-3	-3	-3	9														
	Q	-1	1	0	0	-3	5													
	E	-1	0	0	2	-4	2	5												
	G	0	-2	0	-1	-3	-2	-2	6											
	H	-2	0	1	-1	-3	0	0	-2	8										
	I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4									
	L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4								
15	K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5							
	M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1							
	F	-2	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
	P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7				
	S	1	-1	1	0	-1	0	0	-1	-2	-2	0	-1	-2	-1	4				
20	T	0	-1	0	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
	W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-1	1	-4	-3	-2	11			
	Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7
	V	0	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Substantially homologous proteins and polypeptides are characterized as

5 having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 3) and other substitutions that do not significantly affect the folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker

10 peptide of up to about 20-25 residues, or a small extension that facilitates purification (an affinity tag), such as a poly-histidine tract, protein A [Nilsson *et al.*, *EMBO J.* 4:1075 (1985); Nilsson *et al.*, *Methods Enzymol.* 198:3, (1991)], glutathione S transferase [Smith and Johnson, *Gene* 67:31, (1988)], or other antigenic epitope or binding domain. See, in general Ford *et al.*, *Protein Expression and Purification* 2: 95-107 (1991. DNAs encoding affinity

15 tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

Table 3

Conservative amino acid substitutions

	Basic:	arginine
20		lysine
		histidine
	Acidic:	glutamic acid
		aspartic acid
	Polar:	glutamine
25		asparagine
	Hydrophobic:	leucine
		isoleucine
		valine
	Aromatic:	phenylalanine
30		tryptophan
		tyrosine
	Small:	glycine
		alanine
		serine
35		threonine
		methionine

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis [Cunningham and Wells, *Science* 244: 1081-1085 (1989); Bass 5 *et al.*, *Proc. Natl. Acad. Sci. USA* 88:4498-4502 (1991)]. In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g., ligand binding and signal transduction) to identify amino acid residues that are critical to the activity of the molecule. Sites of ligand-protein interaction can also be determined by analysis of crystal structure as determined by 10 such techniques as nuclear magnetic resonance, crystallography or photoaffinity labeling. See, for example, de Vos *et al.*, *Science* 255:306-312 (1992); Smith *et al.*, *J. Mol. Biol.* 224:899-904, 1992; Wlodaver *et al.*, *FEBS Lett.* 309:59-64 (1992). The identities of essential 15 amino acids can also be inferred from analysis of homologies with related proteins.

Multiple amino acid substitutions can be made and tested using known 15 methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer, *Science* 241:53-57 (1988) or Bowie and Sauer, *Proc. Natl. Acad. Sci. USA* 86:2152-2156 (1989). Briefly, these authors disclose methods for simultaneously randomizing two or more 20 positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display, *e.g.*, Lowman *et al.*, *Biochem.* 30:10832-10837 (1991); Ladner *et al.*, U.S. Patent No. 5,223,409; Huse, WIPO 25 Publication WO 92/06204) and region-directed mutagenesis, Derbyshire *et al.*, *Gene* 46:145 (1986); Ner *et al.*, *DNA* 7:127 (1988).

Mutagenesis methods as disclosed above can be combined with high-throughput screening methods to detect activity of cloned, mutagenized proteins in host cells. Preferred assays in this regard include cell proliferation assays and biosensor-based ligand-binding assays, which are described below. Mutagenized DNA molecules that encode active 30 proteins or portions thereof (*e.g.*, ligand-binding fragments) can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

Using the methods discussed above, one of ordinary skill in the art can prepare 35 a variety of polypeptides that are substantially homologous to SEQ ID NO:2 or allelic variants

thereof and retain the properties of the wild-type protein. As expressed and claimed herein the language, "a polypeptide as defined by SEQ ID NO: 2" includes all allelic variants and species orthologs of the polypeptide.

5 Another embodiment of the present invention provides for a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide of the invention. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. A region of a protein to which an antibody can bind is defined as an "antigenic epitope". See for instance, Geysen, H.M. *et al.*, *Proc. Natl. Acad. Sci. USA* 10 81:3998-4002 (1984).

As to the selection of peptides or polypeptides bearing an antigenic epitope (*i.e.*, that contain a region of a protein molecule to which an antibody can bind), it is well known in the art that relatively short synthetic peptides that mimic part of a protein sequence 15 are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See Sutcliffe, J.G. *et al.* *Science* 219:660-666 (1983). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (*i.e.*, immunogenic epitopes) nor to the amino or carboxyl terminals. 20 Peptides that are extremely hydrophobic and those of six or fewer residues generally are ineffective at inducing antibodies that bind to the mimicked protein; longer soluble peptides, especially those containing proline residues, usually are effective.

Antigenic epitope-bearing peptides and polypeptides of the invention are 25 therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. Antigenic epitope-bearing peptides and polypeptides of the present invention contain a sequence of at least nine, preferably between 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of 30 the invention, containing from 30 to 50 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are useful for inducing antibodies that react with the protein. Preferably, the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (*i.e.*, the sequence includes relatively hydrophilic residues and hydrophobic residues are preferably 35 avoided); and sequences containing proline residues are particularly preferred. All of the polypeptides shown in the sequence listing contain antigenic epitopes to be used according to

the present invention, however, specifically designed antigenic epitopes include the peptides defined by SEQ ID NOs: 27-35.

Polynucleotides, generally a cDNA sequence, of the present invention encode

5 the above-described polypeptides. A cDNA sequence which encodes a polypeptide of the present invention is comprised of a series of codons, each amino acid residue of the polypeptide being encoded by a codon and each codon being comprised of three nucleotides. The amino acid residues are encoded by their respective codons as follows.

10 Alanine (Ala) is encoded by GCA, GCC, GCG or GCT;
Cysteine (Cys) is encoded by TGC or TGT;
Aspartic acid (Asp) is encoded by GAC or GAT;
Glutamic acid (Glu) is encoded by GAA or GAG;
Phenylalanine (Phe) is encoded by TTC or TTT;
15 Glycine (Gly) is encoded by GGA, GGC, GGG or GGT;
Histidine (His) is encoded by CAC or CAT;
Isoleucine (Ile) is encoded by ATA, ATC or ATT;
Lysine (Lys) is encoded by AAA, or AAG;
Leucine (Leu) is encoded by TTA, TTG, CTA, CTC, CTG or CTT;
20 Methionine (Met) is encoded by ATG;
Asparagine (Asn) is encoded by AAC or AAT;
Proline (Pro) is encoded by CCA, CCC, CCG or CCT;
Glutamine (Gln) is encoded by CAA or CAG;
Arginine (Arg) is encoded by AGA, AGG, CGA, CGC, CGG or CGT;
25 Serine (Ser) is encoded by AGC, AGT, TCA, TCC, TCG or TCT;
Threonine (Thr) is encoded by ACA, ACC, ACG or ACT;
Valine (Val) is encoded by GTA, GTC, GTG or GTT;
Tryptophan (Trp) is encoded by TGG; and
Tyrosine (Tyr) is encoded by TAC or TAT.

30 It is to be recognized that according to the present invention, when a cDNA is claimed as described above, it is understood that what is claimed are both the sense strand, the anti-sense strand, and the DNA as double-stranded having both the sense and anti-sense strand annealed together by their respective hydrogen bonds. Also claimed is the messenger
35 RNA (mRNA) which encodes the polypeptides of the present invention, and which mRNA is encoded by the above-described cDNA. A messenger RNA (mRNA) will encode a

polypeptide using the same codons as those defined above, with the exception that each thymine nucleotide (T) is replaced by a uracil nucleotide (U).

The protein polypeptides of the present invention, including full-length

5 proteins, protein fragments (e.g. receptor-binding fragments), and fusion polypeptides can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred.

10 Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, (2nd ed.) (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and Ausubel *et al.*, *ibid.*

15 In general, a DNA sequence encoding a Zcyt07 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be

20 provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

25

To direct a Zcyt07 polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of the protein, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*.

30 The secretory signal sequence is joined to the Zcyt07 DNA sequence in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, *e.g.*, Welch *et al.*, U.S. Patent No. 5,037,743; Holland *et al.*, U.S. Patent No. 5,143,830).

35

Cultured mammalian cells are preferred hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection, Wigler *et al.*, *Cell* 14:725 (1978); Corsaro and Pearson, *Somatic Cell Genetics* 7:603 (1981); Graham and Van der Eb, *Virology* 52:456 (1973), 5 electroporation, Neumann *et al.*, *EMBO J.* 1:841-845 (1982), DEAE-dextran mediated transfection, Ausubel *et al.*, eds., *Current Protocols in Molecular Biology* (John Wiley and Sons, Inc., NY, 1987), and liposome-mediated transfection (Hawley-Nelson *et al.*, *Focus* 15:73 (1993); Ciccarone *et al.*, *Focus* 15:80 (1993)). The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson *et al.*, U.S. 10 Patent No. 4,713,339; Hagen *et al.*, U.S. Patent No. 4,784,950; Palmiter *et al.*, U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 [ATCC No. CRL 1573; Graham *et al.*, *J. Gen. Virol.* 36:59-72 (1977)] and Chinese hamster ovary (e.g. CHO-K1; ATCC No. 15 CCL 61) cell lines. Additional suitable cell lines are known in the art and available from 20 public depositories such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are 25 able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants 30 in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g. hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used.

Other higher eukaryotic cells can also be used as hosts, including insect cells, plant cells and avian cells. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino *et al.*, U.S. Patent No. 5,162,222; Bang *et al.*, U.S. Patent No. 4,775,624; and WIPO publication WO 94/06463. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar *et al.*, *J. Biosci. (Bangalore)* 11:47-58 (1987).

Fungal cells, including yeast cells, and particularly cells of the genus *Saccharomyces*, can also be used within the present invention, such as for producing protein fragments or polypeptide fusions. Methods for transforming yeast cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki *et al.*, U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch *et al.*, U.S. Patent No. 5,037,743; and Murray *et al.*, U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in yeast is the *POT1* vector system disclosed by Kawasaki *et al.*, U.S. Patent No. 4,931,373, which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, *e.g.*, Kawasaki, U.S. Patent No. 4,599,311; Kingsman *et al.*, U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson *et al.*, *J. Gen. Microbiol.* 132:3459-3465 (1986) and Clegg, U.S. Patent No. 4,882,279. Aspergillus cells may be utilized according to the methods of McKnight *et al.*, U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino *et al.*, U.S. Patent No. 5,162,228. Methods for transforming Neurospora are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as

growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell.

5

Within one aspect of the present invention, a novel protein is produced by a cultured cell, and the cell is used to screen for a receptor or receptors for the protein, including the natural receptor, as well as agonists and antagonists of the natural ligand.

10

PROTEIN ISOLATION:

Expressed recombinant polypeptides (or chimeric polypeptides) can be purified using fractionation and/or conventional purification methods and media.

Ammonium sulfate precipitation and acid or chaotropic extraction may be used for

15 fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable anion exchange media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred, with DEAE Fast-Flow Sepharose (Pharmacia, Piscataway, NJ) being particularly preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene 20 beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, 25 hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Methods for binding receptor polypeptides to support media are well known in the art. Selection of a particular method is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, *Affinity* 30 *Chromatography: Principles & Methods* (Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988).

The polypeptides of the present invention can be isolated by exploitation of their *properties*. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins. Briefly, a gel is first charged with divalent metal ions to form a chelate [E. Sulkowski, *Trends in Biochem.* 3:1-7 (1985)]. Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography [*Methods in Enzymol.*, Vol. 182:529-39, "Guide to Protein Purification", M. Deutscher, (ed.), (Acad. Press, San Diego, 1990). Alternatively, a fusion of the polypeptide of interest and an affinity tag (e.g., polyhistidine, maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification. Furthermore, to facilitate purification of the secreted receptor polypeptide, an amino or carboxyl-terminal extension, such as a poly-histidine tag, substance P, FLAG® peptide [Hopp *et al.*, *Bio/Technology* 6:1204-1210 (1988); available from Eastman Kodak Co., New Haven, CT), a Glu-Glu affinity tag [Grussenmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7952-4 (1985)], or another polypeptide or protein for which an antibody or other specific binding agent is available, can be fused to Zcyto7 to aid in purification.

20

Uses

Northern blot analysis of the expression of Zcyto7 reveals that Zcyto7 is specifically expressed in the spinal cord. *In situ* analysis of the spinal cord reveals that this expression is localized in the neurons and dorsal root ganglia. Therefore, Zcyto7 may play a role in the maintenance of spinal cord involving either glial cells or neurons. This indicates that Zcyto7 can be used to treat a variety of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), or dymyelinating diseases including multiple sclerosis. Zcyto7 may also be used to treat sensory neuropathies. The tissue specificity of Zcyto7 expression suggests that Zcyto7 may be a growth and/or maintenance factor in the spinal cord.

Zcyto7 gene's location on chromosome 5 indicates that zcyto7 is a cytokine which can be used to modulate the activities of cells of the immune system. Zcyto7 can also be used as a chemoattractant of neutrophils in the spinal column. This would be useful as an anti-infective for infections in the spinal column. It could also be used to help regulate other cytokines in the spinal cord. Zcyto7 may also be administered to treat peripheral neuropathies

such as Charcot-Marie-Tooth (CMT) disease which is localized to the same chromosomal region of 5q as *Zcyt07*.

The fact that *Zcyt07* inhibits the growth of BAF-3 and TF-1 cells as shown in

5 examples 11 and 12 below indicates that *Zcyt07* can be used to treat autoimmune diseases and possibly such cancers such as leukemias.

The present invention also provides reagents which will find use in diagnostic applications. For example, the *Zcyt07* gene is heavily expressed in the spinal cord. A probe 10 comprising the *Zcyt07* DNA or RNA or a subsequence thereof can be used to determine if the *Zcyt07* gene is present on chromosome 5 or if a mutation has occurred.

The present invention also provides reagents with significant therapeutic value. The *Zcyt07* polypeptide (naturally occurring or recombinant), fragments thereof, 15 antibodies and anti-idiotypic antibodies thereto, along with compounds identified as having binding affinity to the *Zcyt07* polypeptide, should be useful in the treatment of conditions associated with abnormal physiology or development, including abnormal proliferation, e.g., cancerous conditions, or degenerative conditions. For example, a disease or disorder 20 associated with abnormal expression or abnormal signaling by a *Zcyt07* polypeptide should be a likely target for an agonist or antagonist of the *Zcyt07* polypeptide.

In particular, *Zcyt07* can be used to treat inflammation. Inflammation is a result of an immune response to an infection or as an autoimmune response to a self-antigen.

25 Treatment dosages should be titrated to optimize safety and efficacy. Methods for administration include intravenous, peritoneal, intramuscular, subdural, into the spinal fluid or transdermal administration. Pharmaceutically acceptable carriers will include water, saline, buffers to name just a few. Dosage ranges would ordinarily be expected from 0.1 μ g to 1mg per kilogram of body weight per day. Preferably, 1 μ g to 100 μ g per day. However, the 30 doses may be higher or lower as can be determined by a medical doctor with ordinary skill in the art. For a complete discussion of drug formulations and dosage ranges see *Remington's Pharmaceutical Sciences*, 17th Ed., (Mack Publishing Co., Easton, Penn., 1990), and *Goodman and Gilman's: The Pharmacological Bases of Therapeutics*, 9th Ed. (Pergamon Press 1996).

Use of Zcyto7 to Promote Bone and Cartilage Growth

It has been discovered that Zcyto7 stimulates the proliferation of both chondrocytes and osteoblasts as is shown below in Examples 7 and 9 respectively. In

5 addition, Zcyto7 also stimulates the steady state level of glycosaminoglycan present in chondrocyte cultures as shown in Example 8. Thus Zcyto7 can be used to stimulate both bone and cartilage growth in a variety of different therapeutic settings.

Zcyto7 can be implanted in a mammalian body so that the zcyto7 is in contact

10 with osteoblasts such that osteoblast proliferation occurs and bone growth is stimulated. For example, zcyto7 can be placed in a matrix [with or without a bone morphogenic protein (BMP)]. The BMP induces the migration of mesenchymal osteoblast precursors to the site and further induces differentiation of the mesenchymal cells into osteoblast. Zcyto7 will then stimulate the further proliferation of the osteoblasts. A suitable matrix is made up of particles 15 of porous materials. The pores must be of a dimension to permit progenitor cell migration and subsequent differentiation and proliferation. An ideal particle size should be in the range of 70 - 850 mm, preferably 150 - 420mm. The matrix containing the zcyto7 can be molded into a shape encompassing a bone defect. Examples of matrix materials are particulate, demineralized, guanidine extracted, species-specific bone. Other potentially useful matrix 20 materials include collagen, homopolymers and copolymers of glycolic acid and lactic acid, hydroxyapatite, tricalcium phosphate and other calcium phosphates. Zcyto7 can be applied into a matrix at a sufficient concentration to promote the proliferation of osteoblasts, preferably at a concentration of at least 1 μ g/ml of matrix. A solution of zcyto7 can also be injected directly into the site of a bone fracture or defect including areas of bone degeneration 25 to expedite healing of the fracture or defect site. Examples of BMPs and the use of matrices to produce are disclosed in PCT application publication number WO 92/07073, publication No. WO 91/05802, U.S Patent No. 5,645,591 and U.S. Patent No. 5,108,753.

Zcyto7 can be further used to treat osteoporosis by administering a

30 therapeutically effective amount of zcyto7 to an individual. A preferred dosage would be 1 μ g of zcyto7 per kilogram of body weight per day.

As stated above, it has also been determined that zcyto7 can be used to 35 promote the production of cartilage through its ability to stimulate the proliferation of chondrocytes. Zcyto7 can be injected directly into the site where cartilage is to be grown. For example, zcyto7 can be injected directly in joints which have been afflicted with osteoarthritis

or other injured joints in which the cartilage has been worn down. An example of a case in which additional cartilage needs to be grown is shoulders and knees of injured athletes.

Cartilage can also be grown by first removing chondrocytes from an

5 individual, culturing the chondrocytes with zcyto7 so that they proliferate and reimplanting the chondrocytes back into the individual where cartilage needs to be produced.

Zcyto7 can also be used to stimulate the regeneration of dentin or bone which

has been lost due to periodontal disease. To do this, the surrounding tissue should be

10 thoroughly cleaned and a solution of Zcto7 be administered, preferably by injection, into the site in which dentin regeneration is desired.

Antibodies to the zcyto7 polypeptide can be purified and then administered to

a patient. These reagents can be combined for therapeutic use with additional active or inert

15 ingredients, *e.g.*, in pharmaceutically acceptable carriers or diluents along with physiologically innocuous stabilizers and excipients. These combinations can be sterile filtered and placed into dosage forms as by lyophilization in dosage vials or storage in stabilized aqueous preparations. This invention also contemplates use of antibodies, binding fragments thereof or single-chain antibodies of the antibodies including forms which are not
20 complement binding.

The quantities of reagents necessary for effective therapy will depend upon

many different factors, including means of administration, target site, physiological state of the patient, and other medications administered. Thus, treatment dosages should be titrated to

25 optimize safety and efficacy. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in vivo* administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Methods for administration include oral, intravenous, peritoneal, intramuscular, or transdermal administration. Pharmaceutically acceptable carriers will include water, saline, 30 buffers to name just a few. Dosage ranges would ordinarily be expected from 1 μ g to 1000 μ g per kilogram of body weight per day. However, the doses may be higher or lower as can be determined by a medical doctor with ordinary skill in the art. For a complete discussion of drug formulations and dosage ranges see *Remington's Pharmaceutical Sciences*, 17th Ed., (Mack Publishing Co., Easton, Penn., 1990), and *Goodman and Gilman's: The*
35 *Pharmacological Bases of Therapeutics*, 9th Ed. (Pergamon Press 1996).

Nucleic Acid-based Therapeutic Treatment

If a mammal has a mutated or lacks a *Zcyt07* gene, the *Zcyt07* gene can be introduced into the cells of the mammal. In one embodiment, a gene encoding a *Zcyt07* polypeptide is introduced *in vivo* in a viral vector. Such vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. A defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector [Kaplitt *et al.*, *Molec. Cell. Neurosci.*, 2:320-330 (1991)], an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet *et al.*, *J. Clin. Invest.*, 90:626-630 (1992), and a defective adeno-associated virus vector [Samulski *et al.*, *J. Virol.*, 61:3096-3101 (1987); Samulski *et al.* *J. Virol.*, 63:3822-3828 (1989)].

In another embodiment, the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson *et al.*, U.S. Patent No. 5,399,346; Mann *et al.*, *Cell*, 33:153 (1983); Temin *et al.*, U.S. Patent No. 4,650,764; Temin *et al.*, U.S. Patent No. 4,980,289; Markowitz *et al.*, *J. Virol.*, 62:1120 (1988); Temin *et al.*, U.S. Patent No. 5,124,263; International Patent Publication No. WO 95/07358, published March 16, 1995 by Dougherty *et al.*; and *Blood*, 82:845 (1993).

Alternatively, the vector can be introduced by lipofection *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker [Felgner *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987); see Mackey *et al.*, *Proc. Natl. Acad. Sci. USA*, 85:8027-8031 (1988)]. The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cells represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

It is possible to remove the cells from the body and introduce the vector as a naked DNA plasmid and then re-implant the transformed cells into the body. Naked DNA vector for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE

5 dextran, calcium phosphate precipitation, use of a gene gun or use of a DNA vector transporter [see, *e.g.*, Wu *et al.*, *J. Biol. Chem.*, 267:963-967 (1992); Wu *et al.*, *J. Biol. Chem.*, 263:14621-14624 (1988)].

Zcyto7 polypeptides can also be used to prepare antibodies that specifically bind to Zcyto7 polypeptides. These antibodies can then be used to manufacture anti-idiotypic antibodies. As used herein, the term "antibodies" includes polyclonal antibodies, monoclonal antibodies, antigen-binding fragments thereof such as $F(ab')_2$ and Fab fragments, and the like, including genetically engineered antibodies. Antibodies are defined to be specifically binding if they bind to a Zcyto7 polypeptide with a K_a of greater than or equal to $10^7/M$. The affinity of a monoclonal antibody can be readily determined by one of ordinary skill in the art (see, for example, Scatchard, *ibid.*).

Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 20 (Second Edition) (Cold Spring Harbor, NY, 1989); and Hurrell, J. G. R., Ed., *Monoclonal Hybridoma Antibodies: Techniques and Applications* (CRC Press, Inc., Boca Raton, FL, 1982). As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats. The immunogenicity of a Zcyto7 polypeptide may be increased through the use of an adjuvant such as Freund's complete or incomplete adjuvant. A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to Zcyto7 polypeptides. Exemplary assays are described in detail in *Antibodies: A Laboratory Manual*, Harlow and Lane (Eds.), (Cold Spring Harbor Laboratory Press, 1988). Representative examples of such assays include: concurrent 25 immunoelectrophoresis, radio-immunoassays, radio-immunoprecipitations, enzyme-linked immunosorbent assays (ELISA), dot blot assays, inhibition or competition assays, and sandwich assays.

As would be evident to one of ordinary skill in the art, polyclonal antibodies 35 can be generated by inoculating a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, hamsters, guinea pigs and rats with a Zcyto7

polypeptide or a fragment thereof. The immunogenicity of a Zcyto7 polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of Zcyto7 or a portion thereof with an immunoglobulin 5 polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

10 As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as F(ab')₂ and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included. Non-human 15 antibodies may be humanized by grafting non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a " veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper 20 binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced.

25 Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to Zcyto7 protein or peptide, and selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled Zcyto7 protein or peptide). Genes encoding polypeptides having potential Zcyto7 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide 30 sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a

protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art (Ladner et al., US Patent NO. 5,223,409; Ladner et al., US Patent NO. 4,946,778; Ladner et al., US Patent NO. 5,403,484 and Ladner et al., US Patent NO. 5,571,698) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from Clontech (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA) and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the Zcyto7 sequences disclosed herein to identify proteins which bind to Zcyto7.

These “binding proteins” which interact with Zcyto7 polypeptides can be used for tagging cells; for isolating homolog polypeptides by affinity purification; they can be directly or indirectly conjugated to drugs, toxins, radionuclides and the like. These binding proteins can also be used in analytical methods such as for screening expression libraries and neutralizing activity. The binding proteins can also be used for diagnostic assays for determining circulating levels of polypeptides; for detecting or quantitating soluble polypeptides as marker of underlying pathology or disease. These binding proteins can also act as Zcyto7 “antagonists” to block Zcyto7 binding and signal transduction *in vitro* and *in vivo*.

Antibodies can also be generated gene therapy. The animal is administered the DNA or RNA which encodes Zcyto7 or an immunogenic fragment thereof so that cells of the animals are transfected with the nucleic acid and express the protein which in turn elicits an immunogenic response. Antibodies which then are produced by the animal are isolated in the form of polyclonal or monoclonal antibodies.

Antibodies to Zcyto7 may be used for tagging cells that express the protein, for affinity purification, within diagnostic assays for determining circulating levels of soluble protein polypeptides, and as antagonists to block ligand binding and signal transduction *in vitro* and *in vivo*.

Radiation hybrid mapping is a somatic cell genetic technique developed for constructing high-resolution, contiguous maps of mammalian chromosomes [Cox *et al.*, *Science* 250:245-250 (1990)]. Partial or full knowledge of a gene's sequence allows the

designing of PCR primers suitable for use with chromosomal radiation hybrid mapping panels. Commercially available radiation hybrid mapping panels which cover the entire human genome, such as the Stanford G3 RH Panel and the GeneBridge 4 RH Panel (Research Genetics, Inc., Huntsville, AL), are available. These panels enable rapid, PCR based,

5 chromosomal localizations and ordering of genes, sequence-tagged sites (STSs), and other nonpolymorphic- and polymorphic markers within a region of interest. This includes establishing directly proportional physical distances between newly discovered genes of interest and previously mapped markers. The precise knowledge of a gene's position can be useful in a number of ways including: 1) determining if a sequence is part of an existing

10 contig and obtaining additional surrounding genetic sequences in various forms such as YAC-, BAC- or cDNA clones, 2) providing a possible candidate gene for an inheritable disease which shows linkage to the same chromosomal region, and 3) for cross-referencing model organisms such as mouse which may be beneficial in helping to determine what function a particular gene might have.

15

The present invention also provides reagents which will find use in diagnostic applications. For example, the *Zcyt07* gene has been mapped on chromosome 5q31. A *Zcyt07* nucleic acid probe could be used to check for abnormalities on chromosome 5. For example, a probe comprising *Zcyt07* DNA or RNA or a subsequence thereof can be used to determine if

20 the *Zcyt07* gene is present on chromosome 5q31 or if a mutation has occurred. Detectable chromosomal aberrations at the *Zcyt07* gene locus include but are not limited to aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. Such aberrations can be detected using polynucleotides of the present invention by employing molecular genetic techniques, such as restriction fragment length polymorphism (RFLP)

25 analysis, short tandem repeat (STR) analysis employing PCR techniques, and other genetic linkage analysis techniques known in the art [Sambrook *et al.*, *ibid.*; Ausubel, *et. al.*, *ibid.*; Marian, A.J., *Chest*, 108: 255-265, (1995)].

30 *Zcyt07* maps at the 5q31 region which is a "gene cluster" which contains a group of cytokines and cytokine receptors. The cytokines clustered there include IL-3, IL-4, IL-5, IL-13, GM-CSF, and M-CSF. This result authenticates *zcyt07* as a cytokine.

The invention is further illustrated by the following non-limiting examples.

Example 1
Cloning of Zcyto7

Zcyto7 was identified from expressed sequence tag defined (EST) 582069

5 (SEQ ID NO: 3) by its homology to Interleukin-17. The EST 582069 cDNA clone was obtained from a human fetal heart cDNA library from the IMAGE consortium, Lawrence Livermore National Laboratory through Genome Systems, Inc. The cDNA was supplied as an agar stab containing *E. coli* transfected with the plasmid having the cDNA of interest. The plasmid containing the cDNA was streaked out on an LB 100 µg/ml ampicillin and 100 µg/ml

10 methicillin plate. The cDNA insert was sequenced. The insert was determined to be 717 base pairs long with a 180 amino acid open reading frame and a putative 20 amino acid signal peptide.

Example 2
Northern Blot Analysis

15 Human multiple tissue blots 1,2,3 (Clontech) were probed to determine the tissue distribution of Zcyto7. A *Eco*RI/*Not*I fragment containing the entire Zcyto7 coding region was generated from the EST582069 clone and used for the probe. A plasmid prep of EST582069 was prepared from a 5 ml LB 100 µg/ml ampicillin overnight culture at 37° using the QIAprep Spin Miniprep Kit (Qiagen). 12 µl out of 100 µl were digested with 5 µl of H

20 buffer (Boehringer Mannheim), 12.5 units of *Eco*RI (Gibco BRL) and 12.5 units *Not*I (New England Biolabs) in a 50 µl reaction at 37°C for 2 hours. The digest was electrophoresed on a 0.7% TBE agarose gel and the fragment was cut out. To obtain additional material the digest was repeated under the same conditions as above except 24 µl of EST582069 was used in the second digest. The second digest was electrophoresed on a 0.7% TBE agarose gel and the

25 fragment was cut out. The DNA was extracted from both gel slabs with a QIAquick Gel Extraction Kit (Qiagen). 135 ng of this DNA was labeled with P³² using the Multiprime DNA Labeling System (Amersham) and unincorporated radioactivity was removed with a NucTrap Probe Purification Column (Stratagene). Multiple tissue northerns and a human RNA master blot were prehybridized 3 hours with 10 ml ExpressHyb Solution (Clontech) containing 1 mg

30 salmon sperm DNA which was boiled 5 minutes and then iced 1 minute and added to 10 ml of ExpressHyb Solution, mixed and added to blots. Hybridization was carried out overnight at 65°C. Initial wash conditions were as follows: 2X SSC, 0.05% SDS RT for 40 minutes with several changes of solution then 0.1X SSC, 0.1% SDS at 50°C for 40 minutes, 1 solution change. Blots were then exposed to film at -80°C for 5 hours. There was cross

35 hybridization/background so blots were further washed at 55°C then 65°C with 0.1% X SSC,

0.1% SDS for 1 hour each. Spinal cord showed very high expression of *Zcyt07* mRNA and trachea showed weak expression of mRNA. The transcript size was approximately 0.75 kb.

Example 3

Chromosomal Assignment and Placement of *Zcyt07*

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Zcyt07 was mapped to chromosome 5 using the commercially available "GeneBridge 4 Radiation Hybrid Panel" (Research Genetics, Inc., Huntsville, AL). The GeneBridge 4 Radiation Hybrid Panel contains PCRable DNAs from each of 93 radiation hybrid clones, plus two control DNAs (the HFL donor and the A23 recipient). A publicly 10 available WWW server (<http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl>) allows mapping relative to the Whitehead Institute/MIT Center for Genome Research's radiation hybrid map of the human genome (the "WICGR" radiation hybrid map of the human genome) which was constructed with the GeneBridge 4 Radiation Hybrid Panel.

15 For the mapping of *Zcyt07* with the "GeneBridge 4 RH Panel", 20 \pm 1 reactions were set up in a PCRable 96-well microtiter plate (Stratagene, La Jolla, CA) and used in a "RoboCycler Gradient 96" thermal cycler (Stratagene). Each of the 95 PCR reactions consisted of 2 \pm 1 10X KlenTaq PCR reaction buffer (CLONTECH Laboratories, Inc., Palo Alto, CA), 1.6 \pm 1 dNTPs mix (2.5 mM each, PERKIN-ELMER, Foster City, CA), 1 \pm 1 sense 20 primer SEQ ID NO:4, 1 \pm 1 antisense primer SEQ ID NO:5, 2 \pm 1 "RediLoad" (Research Genetics, Inc., Huntsville, AL), 0.4 \pm 1 50X Advantage KlenTaq Polymerase Mix (Clontech Laboratories, Inc.), 25 ng of DNA from an individual hybrid clone or control and x \pm 1 ddH₂O for a total volume of 20 \pm 1. The reactions were overlaid with an equal amount of mineral oil and sealed. The PCR cycler conditions were as follows: an initial 1 cycle 5 minute 25 denaturation at 95°C, 35 cycles of a 1 minute denaturation at 95°C, 1 minute annealing at 52°C and 1 minute extension at 72°C, followed by a final 1 cycle extension of 7 minutes at 72°C. The reactions were separated by electrophoresis on a 3% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME).

30 The results showed that *Zcyt07* maps 490.89 cR from the top of the human chromosome 5 linkage group on the WICGR radiation hybrid map. Relative to the centromere, its nearest proximal marker was D5S413 and its nearest distal marker was WI-5208. The use of surrounding markers position *Zcyt07* in the 5q31.3-q32 region on the integrated LDB chromosome 5 map (The Genetic Location Database, University of 35 Southampton, WWW server: http://cedar.genetics.soton.ac.uk/public_html/).

Example 4Construction of Zcyto7 Expression Vectors

Two Zcyto7 construction vectors were made in a FLAG amino acid sequence (SEQ ID NO: 10) was inserted onto the N-terminal or C-terminal ends of the Zcyto7 polypeptide. For the construction in which the FLAG amino acid sequence was attached to the N-terminus of Zcyto7, a 473 bp Zcyto7 PCR DNA fragment was generated with 1 μ l of a $\frac{1}{10}$ dilution of the EST582069 plasmid prep of Example 2 and 20 picomoles (pm) of primer SEQ ID NO: 6 and 20 pm primer SEQ ID NO: 7. The PCR reaction was incubated at 94°C for 1 minute, and then run for 5 cycles each individual cycle being comprised of 20 seconds at 94°C and 2 minutes at 64°C. This was followed by 22 cycles each cycle being comprised of 20 seconds at 94°C and 2 minutes at 74°C. The reaction was ended with an incubation for 10 minutes at 74°C. 50 μ l of the PCR reaction mixture was digested with 30 units of *Bam*H1 (Boehringer Mannheim) and 120 units of *Xho*1 (Boehringer Mannheim) for 2 hours at 37°C. The digested reaction mixture was electrophoresed on a 1% TBE gel; the DNA band was excised with a razor blade and the DNA was extracted from the gel with the Qiaquick® Gel Extraction Kit (Qiagen). The excised DNA was subcloned into plasmid nfpzp9 which had been cut with *Bam* and *Xho*. Nfpzp9 is a mammalian cell expression vector comprising an expression cassette containing the mouse metallothionein-1 promoter, a sequence encoding the tissue plasminogen activator (TPA) leader, then the FLAG peptide (SEQ ID NO:10), then multiple restriction sites. These were followed by the human growth hormone terminator, an *E. coli* origin of replication and a mammalian selectable marker expression unit containing the SV40 promoter, enhancer and origin of replication; a dihydrofolate reductase gene (DHFR) and the SV40 terminator.

25

For the construction of the zcyto7 gene in which a C-terminus FLAG was inserted onto the C-terminus of the zcyto7 polypeptide, a 543 bp zcyto7 PCR fragment was generated with 1 μ l of $\frac{1}{10}$ dilution of the EST582069 plasmid preparation described in Example 1 and 20 pm each of primers SEQ ID NO: 8 and SEQ ID NO: 9. The PCR reaction was incubated at 94°C for 1 minute, then run for 5 cycles, each cycle being comprised 20 seconds at 94°C and 2 minutes at 55°C. This was followed by 22 cycles each cycle comprised of 20 seconds at 94°C and 2 minutes at 74°C. The reaction was ended with a final 10 minute extension at 74°C. The entire reaction mixture was run on a 1% TBE gel and the DNA was cut out with a razor blade and the DNA was extracted using the QIAQUICK™ gel extraction kit. 20 μ l out of the recovered 35 μ l digested with 10 units of *Bam*H1 (Boehringer Mannheim) and 10 units of *Eco*R1(Gibco BRL) for 2 hours at 37°C. The digested PCR

mixture was electrophoresed on a 1% TBE gel. The DNA band was cut out with a razor blade and the DNA was extracted from the gel using the QIAquick® Gel Extraction Kit (Qiagen).

The extracted DNA was subcloned into plasmid cfpzp9 which had been cut with *Eco*R1 and *Bam*H1. Plasmid cfpzp9 is a mammalian expression vector containing an expression cassette

5 having the mouse metallothionein-1 promoter, multiple restriction sites for insertion of coding sequences, a sequence encoding the FLAG peptide, SEQ ID NO:10, a stop codon, a human growth hormone terminator, an *E. coli* origin of replication, a mammalian selectable marker expression unit having an SV40 promoter, enhancer and origin of replication, a DHFR gene and the SV40 terminator.

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Using antibodies to the FLAG polypeptides, one can separate the FLAG-tagged Zcyt07 polypeptides from a cell supernatant liquid.

Example 5

15

Cloning of Murine Zcyt07

Mouse Zcyt07 was identified from EST 660242 SEQ ID NO:14 by its homology to human Zcyt07. The cDNA clone was obtained from Lawrence Livermore National Laboratory through Genome Systems from a murine embryo cDNA library in which

20 the embryos were between 13.5 and 14.5 days old. The cDNA was supplied as an agar stab containing *E. coli* transfected with the plasmid having the cDNA of interest and then streaked out on an LB 100 µg/ml ampicillin, 25 µg/ml methicillin plate. The cDNA insert in EST660242 was sequenced. The insert was determined to be 785 base pairs with an open reading frame of 180 amino acids and a putative 20 amino acid signal peptide. The sequences

25 are defined by SEQ ID NO:11 and SEQ ID NO:12.

Example 6

Tissue Distribution of Murine Zcyt07

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Mouse Multiple Tissue Northern Blot (Clontech, Palo Alto, CA), mouse northern dot blot (Clontech), a mouse embryo northern blot, and a mouse spinal cord dot blot were probed to determine the tissue distribution of murine Zcyt07.

35 The mouse embryo RNA were isolated from mouse embryos which were 6 to nine days from the date of fertilization using the POLY (A) PURE® mRNA isolation kit (Ambion). 100 mg of each mouse embryo was lysed in 1 ml of lysis buffer, homogenized and

processed in batch method according to the manufacturer's protocol. For the northern blot, 2 µg of RNA was loaded on 1.5% agarose, 2.2M formaldehyde gel. The gel was run at 60V for four hours and 30 minutes. The RNA was transferred overnight onto a Nytran membrane which had been pre-wetted in 20 X SSC. The RNAs were crosslinked onto the membrane by

5 UV light and baked at 80°C for 1 hour.

The mouse spinal cord RNA was also prepared with the POLY (A) PURE® mRNA isolation kit (Ambion). The mouse spinal cord dot blot was made by spotting a dot with 1, 2 and 3 µl of RNA at 1µg RNA/µl concentration onto Nytran membrane.

10

A *Not1/EcoRI* fragment containing the entire *Zcyt07* coding region was generated from the clone containing SEQ ID NO: 12 (hereinafter referred to as the SEQ ID NO:12 clone) and was used for the probe. A plasmid prep of the SEQ ID NO:12 clone was prepared from a 5 ml LB 100 µg/ml ampicillin overnight culture at 37°C using the QIAPREP 15 SPIN MINIPREP kit (Qiagen). 4.66 µg were digested with 8 µl of high buffer (Boehringer Mannheim), 20 units of *Not1* (Biolabs) and 20 units of *EcoRI* (Gibco BRL) in a 80 µl reaction at 37°C for 2 hours. The digest was electrophoresed on a 1.0% TBE gel and the fragment was cut out. The DNA was extracted from the gel slab with a QIAQUICK® gel extraction kit (Qiagen). 98.8 ng of this fragment was labeled with P³² using the 20 MULTIMPRIME® DNA labeling system (Amersham) and unincorporated radioactivity was removed with a NUCTRAP® probe purification column (Stratagene).

The two northern preps and the two dot blot preps were prehybridized for 3 hours at 65°C as follows. 1 mg of salmon sperm was boiled 5 minutes, iced 1 minute, mixed 25 with 10 ml of EXPRESSHYB® solution and added to the blots. Hybridization was carried out overnight at 65°C. Initial wash conditions were as follows: 2 X SSC, 0.1% SDS for 40 minutes at room temperature then 0.1 X SSC, 0.1% SDS for 40 minutes at 50°C. Blots were exposed to film overnight at -80°C. The northern blots and the mouse dot blot were further washed with 0.1% X SSC, 0.1% SDS at 60°C to remove background. The mouse spinal cord 30 dot blot was washed again at higher stringency with 0.1 X SSC, 0.1% SDS at 65°C to confirm the earlier results.

Results: Mouse *Zcyt07* expression was seen in the spinal cord, submaxillary gland and epididymis. Mouse embryo showed expression of *Zcyt07* starting on day 12, peaking at day 35 16 and ending day 17 from the date of fertilization. The transcript size was approximately 1 kb.

Example 7Proliferation of Chondrocytes Using Zcyto7

5 A chondrocyte proliferation assay was done to determine the effect that Zcyto7 would have on chondrocyte proliferation. The assay was done with 20% confluent cultures. As a control vehicle, bovine serum albumin was added to a culture of chondrocytes instead of Zcyto7. The assay measured the 3H-thymidine incorporation of nascent DNA in the chondrocytes, Wahl *et al.*, *Mol. Cell. Biol.* 8:5016-5025 (1988).

10 **Results:** A 3.5 - 9 fold stimulation of primary chondrocyte proliferation was seen upon exposure of the chondrocyte cultures to 1 μ g/ml of zcyto7. Chondrocyte stimulation by zcyto7 was seen with multiple preparations of the protein and was seen across species lines. It contrast to this, the control experiment using BSA resulted in no stimulation of chondrocytes.

15 Example 8
Production of Glycosaminoglycan by Zcyto7-treated Chondrocytes

A 20% confluent culture of chondrocytes was prepared and zcyto7 was applied

20 at a concentration of 1 μ g/ml. In a second experiment in addition to zcyto7, IL-1 β was applied to the cell culture. In a control group BSA was added to the chondrocyte culture. The level of glycosaminoglycan (GAG) production by the chondrocyte culture was then determined using a 1,9-dimethylmethylen blue dye binding assay, Fardale *et al.*, *Biochem. Biophys. Acta* 888:173-177 (1987).

25 **Results:** Chondrocytes which were cultured with zcyto7 showed a 50% increase in the steady state presence of GAG in the chondrocyte culture. Moreover, when the chondrocytes were co-cultured with both zcyto7 and interleukin-1 β (IL-1) the GAG production by the chondrocytes increased 2.5 fold as compared with culturing of the chondrocytes with either zcyto7 or IL-1 β alone. While the cultured cells to which BSA was added showed no increased production of GAG.

Example 9
Osteoblast Stimulation by Zcyto7

The CCC4 cell line is an osteoblast-like cell line derived from p53 knockout mice. The CCC4 line was transfected with a plasmid containing an inducible serum response element (SRE) driving the expression of luciferase. The stimulation of the SRE and thus the expression of luciferase indicates that the chemical entity is likely to stimulate osteoblasts.

CCC4 cells were cultured in the presence of 1 μ g of zcyto7/ml of culture medium. As a control BSA, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) were each added to different cultures of CCC4 cells. BSA was a negative control and FGF and PDGF were positive controls as they are known to promote osteoblast proliferation. Luciferase activity was detected by addition of 40 μ l of Promega luciferase substrate using a 2 second integrated read on Labsystems LUMINOSCAN™.

15

Results

Zcyto7 as well as, FGF and PDGF stimulate the expression of luciferase in this assay indicating that they stimulate osteoblasts. The BSA vehicle control was negative in this assay.

20

Example 10
Effect of Zcyto7 on the Growth of Fibroblasts

Confluent cultures of human dermal, lung, and fetal lung fibroblasts were inoculated with Zcyto7 to determine the effects of Zcyto7 on the growth of fibroblasts. FGF was used as a positive control and BSA as a negative control vehicle.

Results: Zcyto7 had no effect on the growth of fibroblasts.

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Example 11
Effect of Zcyto7 on the Growth of BaF3 Cells

BaF3 cells, a murine pre-B cell line dependent on IL-3 to proliferate, were washed several times with base medium and then plated in a 96-well plate each well contained approximately 5500 cells/well. The cells were treated with either 1 μ g/ml of Zcyto7 or 1-2 μ g/ml of IL-3 or with a combination of both Zcyto7 and IL-3. Also in a separate

experiment 0.1-10ng/ml of TGF β was added to the wells instead of Zcyto7. After incubation of the assay plate at 37°C and 5% CO₂ for 3-6 days, 20 μ l of ALAMAR blue was added to each well and the plate is incubated at 37° C for 15-24 hours. The plate was then read with a fluorometer with excitation wavelength of 544m and emission wavelength of 590m. The 5 assay was also scored by eye for stimulation or inhibition of cell proliferation prior to the addition of the ALAMAR blue. The base medium contained RPMI 1640 + 10% HIA-FBS + L-glutamine + Na pyruvate.

Results: Zcyto7 and TGF β significantly inhibited the IL-3 driven proliferation of BaF3 cells. 10 However, when neutralizing antibodies to TGF β were added in along with the Zcyto7, the Zcyto7 inhibition of the proliferation of the BaF3 cells was eliminated.

Example 12

Effect of Zcyto7 on the Growth of TF-1 Cells

15 TF-1 cells, a human leukemia cell line which is GM-CSF or IL-1 β dependent, were washed several times with base medium and then plated in a 96-well plate each well containing approximately 7000 cells/well. The cells were co-cultured with 1 μ g/ml of Zcyto7 and 100-200pg/ml of IL-1 β . Also in a separate experiment TGF β was added to the wells 20 instead of Zcyto7. After incubation of the assay plate at 37°C and 5% CO₂ for 3-6 days, 20 μ l of ALAMAR blue was added to each well and the plate is incubated at 37° C for 15-24 hours. The plate is then read with a fluorometer with excitation wavelength of 544nm and emission wavelength of 590nm. The assay was also scored by eye for stimulation or inhibition of cell proliferation prior to the addition of the ALAMAR blue. The base medium contained RPMI 25 1640 + 10% HIA-FBS + L-glutamine + Na pyruvate.

Results: The IL-1 β stimulation of TF-1 cells is inhibited by both Zcyto7 and TGF- β . The concentration of Zcyto7 at which inhibition of proliferation occurred was greater than 30 200ng/ml; and the concentration of TGF- β at which inhibition of proliferation occurred was about 50pg/ml.